2. The partial activation which occurs in hypotonic media takes place very rapidly, does not show a further increase if the time of exposure at 0° is prolonged and is not reversed by a return to isotonicity.

3. Inorganic univalent salts afford only transient osmotic protection. In contrast, part of the sucrose can be replaced in the medium by an iso-osmolar quantity of β-glycerophosphate, with no adverse effect on the stability of the granules. 2:4-Dinitrophenol is without significant influence on the activation of acid phosphatase.

4. The activation which occurs when the granules are incubated at 37° in 0·25 M sucrose is much more rapid at pH 5 than at pH 6·1.

5. The internal enzyme appears to be entirely inaccessible to external glycerophosphate at pH 5, but seems to contribute significantly to the free activity of intact preparations as the pH is raised, or as the temperature is increased at pH 6·1.

6. The results obtained afford additional support for the sac-like representation of the acid phosphatase-containing granules arrived at in earlier work. It is pointed out that a limited degree of permeability of the membrane to glycerophosphate remains compatible with perfect osmotic protection by this compound, as long as its rate of inward diffusion does not exceed the rate at which it can be hydrolysed within the granules and eliminated in the form of glycerol and inorganic phosphate.

These investigations have been supported by grants from the 'Centre National de Recherches sur la Croissance normale et pathologique', the Rockefeller Foundation and the Lilly Research Laboratories.

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Tissue Fractionation Studies

4. COMPARATIVE STUDY OF THE BINDING OF ACID PHOSPHATASE, β-GLUCURONIDASE AND CATHEPSIN BY RAT-LIVER PARTICLES

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In a study of the β-glucuronidase of mouse liver, Walker (1952) has described a number of observations which resemble in a striking fashion the findings made in this laboratory on the unspecific acid phosphatase of rat liver (Berthet & Duve, 1951; Berthet, Berthet, Appelmans & Duve, 1951; Appelmans & Duve, 1955). Like acid phosphatase, β-glucuronidase occurs largely in particulate form; it is partly unreactive when in the bound state and is rendered simultaneously soluble and fully active by all the treatments which have been found to cause a similar release of acid phosphatase. After it was verified in preliminary experiments that the findings of Walker (1952) also apply to the β-glucuronidase of rat liver, it was decided to make a parallel study of the release of the two enzymes, using the methods previously worked out for the investigations on acid phosphatase.

In a search for other enzymes of the same type in rat liver, it was found that cathepsin, whose intracellular distribution recalls in some respects that of acid phosphatase (Maver & Greco, 1951), also exhibits the same general pattern of behaviour. Accordingly, the release of this enzyme was also studied in a number of comparative experiments with acid phosphatase. These studies, which have been reported elsewhere in condensed form (Duve, Gianetto, Appelmans & Wattiaux, 1953), have revealed a close parallelism, both qualitative and quantitative, in the manner of release of the three enzymes.

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EXPERIMENTAL

Methods

All the experiments were performed on twice-washed mitochondria fractions, isolated in 0·25M sucrose from the livers of rats fasted for 12 hr., by the method previously described (Appelmans & Duve, 1955). The preparations were subjected to the treatment chosen to cause partial activation, following which the free enzymic activities were determined by incubation for 10 min. at 37° in the presence of the substrate and of sufficient sucrose to make the concentration of this sugar 0·25M in the mixture. Total activities were measured in a similar manner on preparations previously exposed for 3 min. to the action of the Waring Blendor, usually after a tenfold dilution in distilled water. Care was taken to prevent overheating during the blendor treatment.

Acid phosphatase. This was determined as before, by measuring the amount of inorganic P set free in the presence of 0·05M β-glycerophosphate and 0·05M acetate buffer, pH 5. The total volume of the incubation mixture was 2 ml.

β-Glucuronidase. This was assayed by an adaptation of the method of Talalay, Fishman & Huggins (1946) as modified by Kerr & Levvy (1951). The incubation was performed in a total volume of 2 ml., containing 0·00125M phenolphthalein glucuronide and 0·075M acetate buffer, pH 5·2. The reaction was stopped by adding 6 ml. of a solution containing 0·133M glycine, 0·067M-NaCl and 0·083M-Na_2CO_3, pH 10·7. The mixture was clarified by filtration or high-speed centrifuging and its phenolphthalein content determined in a Klett-Summerson photoelectric colorimeter, using a green light-filter (no. 54). A blank containing all the ingredients with the exception of phenolphthalein glucuronide was run in an identical manner. The contribution of the substrate itself to the light absorption was estimated separately; it was always very low. The liberation of phenolphthalein, as measured in this way, proceeds linearly with time and is proportional to the amount of enzyme added. Sucrose is without effect on the determination of phenolphthalein but causes a 25–30% inhibition of enzyme activity at 0·25M concentration. Since all assays were performed in isotonic sucrose, the inhibition was assumed to be constant in all cases and was not taken into account.

Cathepsin. Special difficulties were encountered in an attempt to adapt the cathepsin assay of Anson (1937) to the needs of the present study. In the first place, tests for free activity had to be carried out at pH 5 and for not longer than 10 min., because the granules are too unstable at a lower pH and do not resist longer incubation at 37°. However, the enzyme is only one-quarter as active at pH 5 as at pH 3·6. Since, in addition, the cathepsin activity of rat liver is already fairly weak and often only a fraction of the total enzyme was measured, reliable assays would have required larger amounts of material than was practical in most cases. A second difficulty arose from the necessary presence of large quantities of sucrose. This sugar is progressively hydrolysed in the trichloroacetic acid which is used to stop the reaction, giving rise to hexoses which cause a significant reduction of the Folin–Ciocalteu reagent. Finally, it was found that the relationship between activity and duration of incubation or enzyme concentration, while perfectly linear, does not however start exactly at the origin of the axes. Apparently, a slight addition to the blank occurs during the initial stage of the incubation. The shortest time required to attain linearity was found to be 2 min.

After exploring various possibilities, the following procedure was finally adopted. The incubation mixture contained, in a total volume of 3 ml., 0·00026M haemoglobin and 0·17M acetate buffer pH 5, in addition to the enzyme and 0·25M sucrose. Duplicate samples were incubated at 37° for 2 and 10 min., respectively. The reaction was stopped by adding 5 ml. of ice-cold 0·3M trichloroacetic acid; the mixture was cooled in ice immediately and filtered in the ice box. Aromatic degradation products were measured on the filtrates by means of the Folin–Ciocalteu reagent, with tyrosine as standard, as in the original method of Anson (1937). The difference between the readings found with the two samples served to calculate the tyrosine equivalent set free in 8 min. When applied carefully, this method furnishes a reliable assay despite the presence of sucrose. However, the fact that the measured activities were usually low rendered a certain degree of inaccuracy inevitable.

Materials

Phenolphthalein glucuronide. This was isolated as the crystalline cinchonidine salt from the urine of rabbits injected with phenolphthalein phosphate, according to a modification of the method of Talalay *et al.* (1946), supplied to us by the Rowett Research Institute, Bucksburn, Aberdeenshire. The recrystallized product contained neither inorganic phosphate nor free phenolphthalein and was found to yield the theoretical amount of phenolphthalein after acid hydrolysis. The substrate was prepared by extracting the free acid with ethyl acetate, evaporating to dryness, and redissolving in water together with enough NaOH to bring the pH to 5·2.

Crystalline haemoglobin. This was prepared from washed ox erythrocytes, by a method involving dialysis against saturated $(NH_4)_2SO_4$. The crystals were redissolved in water and dialysed exhaustively against distilled water. The concentration of the resulting solution was measured by the reduced pyridine haemochromogen method (Duve, 1948).

Protein. This was determined by the technique of Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

As will be obvious from the results reported below, the bound forms of β-glucuronidase and cathepsin share the main properties described previously for particulate acid phosphatase; largely unreactive towards their respective substrates under the conditions of the free activity assays, they are both released in soluble form and activated 5- to 10-fold by the treatments which liberate acid phosphatase. Once these points were established, it seemed of interest to ascertain to what extent the observed similarities hold true on a quantitative basis. Advantage was taken for this purpose of the various means known to elicit a partial and graded activation of acid phosphatase. The release of the other two hydrolases was studied under the same conditions, in experiments which always included simultaneous measurements of acid phosphatase, to

serve as a basis of comparison. For practical reasons, a greater number of experiments were made on β-glucuronidase than on cathepsin.

Mechanical disruption

By running the Waring Blendor at 130v instead of 220v, it was possible to activate preparations in a graded manner by means of this instrument. Fig. 1 shows the results of two experiments in which parallel measurements of free activity were made after increasing times of exposure to the Waring Blendor. It is seen that the release of β-glucuronidase and cathepsin both follow that of acid phosphatase very closely.

Activation by freezing and thawing

As illustrated in Fig. 2, a similar parallelism obtains when repeated freezing and thawing is used to activate the preparations.

Osmotic activation

The method used to study the osmotic activation of the three enzymes was based on the experiments of Appelmans & Duve (1955). Granules were exposed for 15 min. at 0° to media of decreasing sucrose concentration, following which samples of each dilution were added to a substrate mixture containing enough sucrose to make the final concentration of this sugar 0·25 M in the enzyme assay. The results of two experiments of this type are shown in Figs. 3 and 4.

Activation by salts

As shown in earlier experiments (Berthet *et al.* 1951; Appelmans & Duve, 1955), the acid phosphatase-containing granules do not retain their integrity when kept at 0° in isotonic NaCl or KCl, whereas they remain perfectly stable under the same conditions in 0·25M sucrose. The data in Fig. 5 indicate that the same is true for bound β-glucuronidase. In this experiment, the free acid phosphatase and β-glucuronidase activities were measured at regular intervals on preparations kept at 0° in 0·25M sucrose and in 0·15M-NaCl, respectively. Following the directions of Appelmans & Duve (1955), the substrates added to each sample contained enough sucrose and NaCl to make the respective concentrations of these substances 0·25M and 0·15M in all cases, during the enzyme assay.

Thermal activation

Fig. 6 summarizes the results of three experiments, in which the preparation was activated in 0·25M sucrose by incubation at 37° and pH 5. Since the three experiments gave almost identical results, only the mean values are given.

Chemical separation

Once released, acid phosphatase and β-glucuronidase behave as distinct soluble proteins. To verify this fact a soluble extract was prepared by

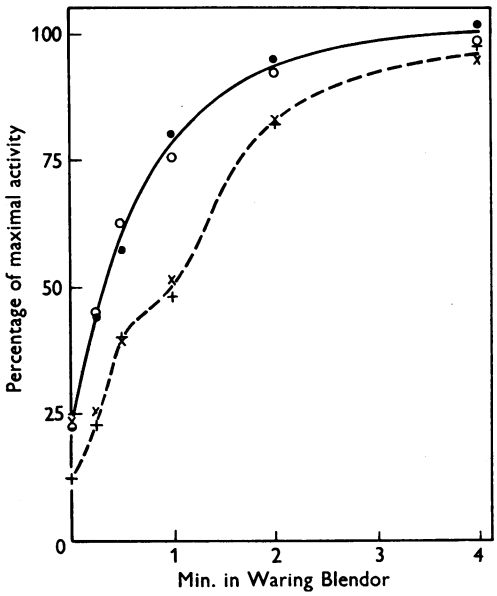

Fig. 1. Activation of acid phosphatase and β-glucuronidase or cathepsin by disintegration of particles in a Waring Blendor. Preparations diluted in 0·25M sucrose and treated in a Waring Blendor running at low speed (130v instead of 220v) for increasing periods of time. Expt. 1, acid phosphatase (●) and β-glucuronidase (○); Expt. 2; acid phosphatase (×) and cathepsin (+).

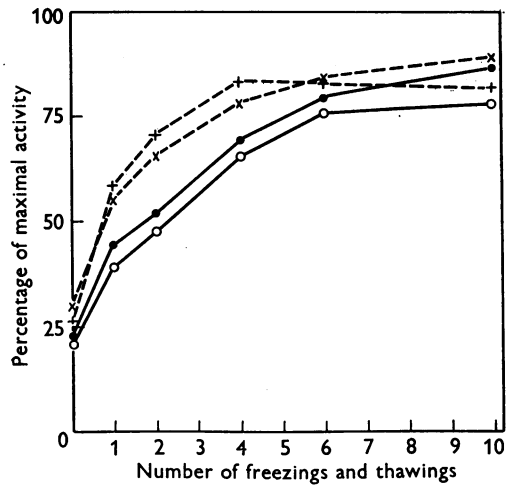

Fig. 2. Activation of acid phosphatase and β-glucuronidase or cathepsin by repeated freezing and thawing. Preparations diluted in 0·25M sucrose and frozen and thawed an increasing number of times. Expt. 1; acid phosphatase (●) and β-glucuronidase (○); Expt. 2; acid phosphatase (×) and cathepsin (+).

treating a suspension of granules in the Waring Blendor in the presence of 0·1 M acetate buffer, pH 5, incubating the mixture 30 min. at 37° and centrifuging at high speed. The supernatant was dialysed against distilled water, clarified by centrifuging and fractionated with ammonium sulphate in the presence of 0·1 M acetate buffer, pH 5. Each precipitate was collected, dialysed free from ammonium sulphate and analysed for acid phosphatase, β-glucuronidase and protein. The specific activities of each fraction are summarized graphically in Fig. 7.

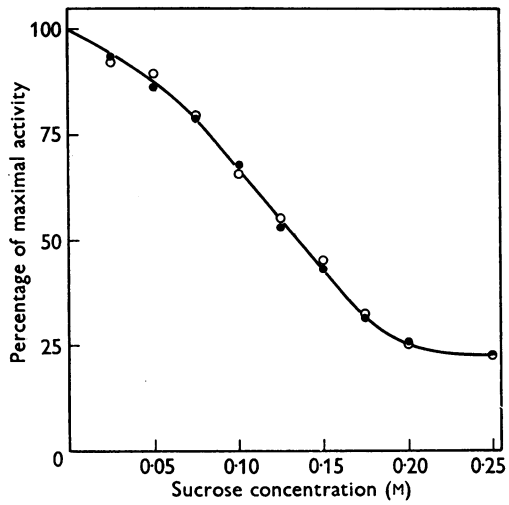

Fig. 3. Activation of acid phosphatase and β-glucuronidase in hypotonic media. Preparations exposed for 15 min. at 0° to sucrose concentration indicated on abscissa. Enzyme assays carried out in 0·25 M sucrose. ●, acid phosphatase; ○, β-glucuronidase.

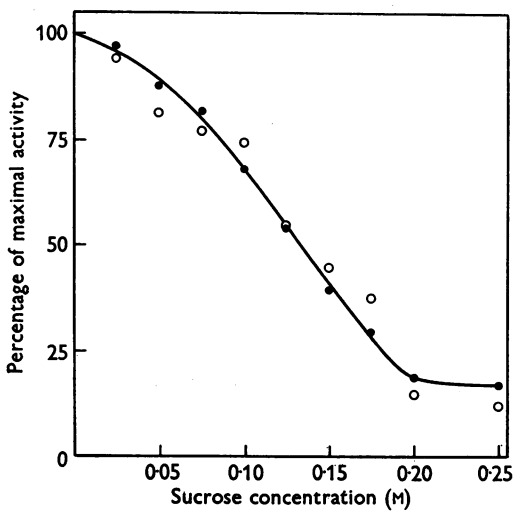

Fig. 4. Activation of acid phosphatase and cathepsin in hypotonic media. Experimental conditions as in Fig. 3. ●, acid phosphatase; ○, cathepsin.

The method outlined above can provide a useful starting-point for the purification of acid phosphatase. In this case the blender extract is incubated at 50°, resulting in a greater denaturation of inactive proteins. By collecting the fraction precipitating between 40 and 75% saturation of ammonium sulphate, and refractionating twice between 58 and 67% saturation, a 300-fold purification over the original tissue is easily achieved.

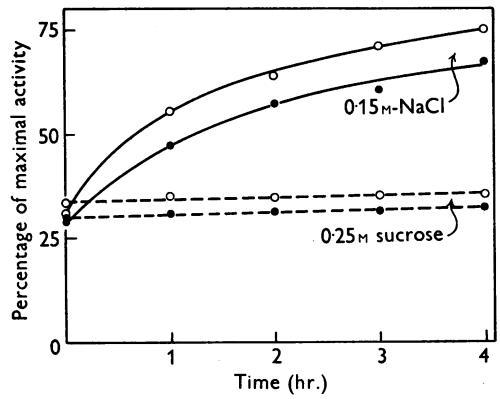

Fig. 5. Activation of acid phosphatase and β-glucuronidase in 0·15 M-NaCl. Preincubation at 0° in 0·25 M sucrose or in 0·025 M sucrose and 0·15 M-NaCl. All enzyme assays carried out in 0·25 M sucrose and 0·15 M-NaCl. ●, acid phosphatase; ○, β-glucuronidase.

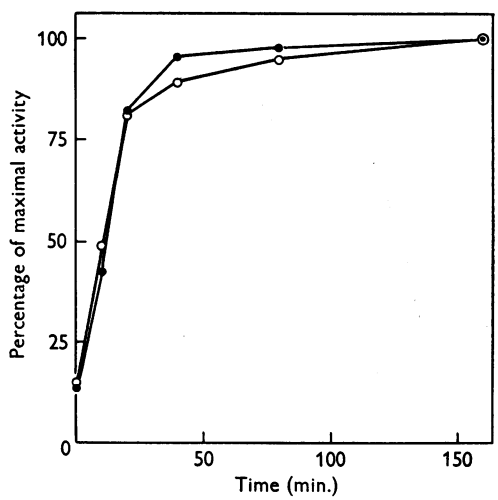

Fig. 6. Thermal activation at pH 5 of acid phosphatase and β-glucuronidase. Preincubation at 37° in 0·25 M sucrose and acetate buffer, pH 5. Mean of three experiments: 1 and 2 in 0·01 M acetate buffer; 3, same preparation as in 2, but in 0·1 M acetate buffer. Acid phosphatase and β-glucuronidase both assayed at pH 5. The values at 169 min. are taken as 100% activity. ●, acid phosphatase; ○, β-glucuronidase.

DISCUSSION

The present investigations were prompted largely by the similarities which were noted between the results of Walker (1952) on the β-glucuronidase of mouse liver and the observations made in this laboratory (Berthet & Duve, 1951; Berthet *et al.* 1951; Appelmans & Duve, 1955) on the acid phosphatase of rat liver. These similarities become even more striking when the two enzymes are studied simultaneously on the same material by means of identical techniques. In addition, they have been found to extend also to cathepsin, another hydrolase with an acid pH optimum, for which this type of behaviour had not been described before.

In the case of bound acid phosphatase, previous investigations have shown that its properties can be entirely explained by the assumption that the enzyme is retained within the granules by a membrane-like barrier showing a low degree of permeability towards β-glycerophosphate. Even though the evidence available for the other two hydrolases is somewhat less complete, it seems fairly safe to conclude, by analogy, that they are likewise enclosed within sac-like granules, surrounded by a membrane impermeable to their respective substrates.

In examining the data on a quantitative basis, one is struck by the close correlations which have been found in the greatest number of experiments. Slight discrepancies have been observed occasionally, but their significance is doubtful in view of the numerous causes of error which are likely to affect experiments of this type. Irregularities, such as occur in some cathepsin experiments, are easily accounted for by the difficulties which complicate the measurements of free activity with this enzyme. These difficulties have been stressed in the section on methods. On the other hand, the cases in which one of the curves appears to be shifted somewhat with respect to the other could arise either from unequal contaminations of the granules by adsorbed enzyme or from errors in the estimation of the total activities, due for instance to a slight denaturation of one of the enzymes in the course of the blender treatment. This interpretation is supported by the fact that even the initial free activities differ in the two experiments in which such shifts have been observed (β-glucuronidase experiment of Figs. 2 and 5).

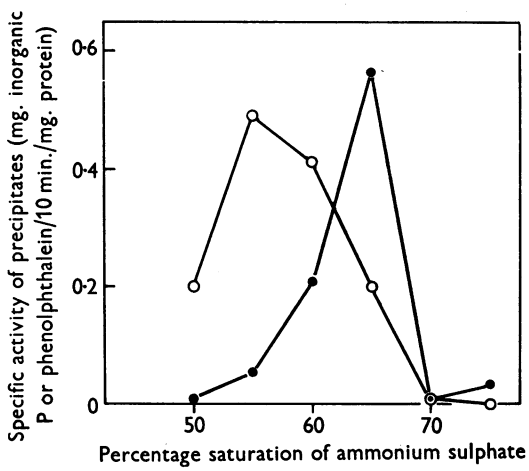

Fig. 7. Separation of acid phosphatase and β-glucuronidase by ammonium sulphate fractionation. Specific activities of fractions precipitated from a soluble extract of large granules at increasing ammonium sulphate concentrations. ●, acid phosphatase; ○, β-glucuronidase.

The various causes of error referred to above could mask dissociations as well as correlations, and it is therefore fair to state that the evidence produced is compatible with the view either that the three enzymes are released in an identical manner in all the circumstances investigated or that the processes are only grossly similar and distinguished by slight but significant differences. It seems, however, justified to attach more weight to the experiments in which almost perfect correlations were observed, since they are more numerous and offer no indication, as do the others, of having been complicated by an artifact.

Turning now to the actual interpretation of the results, it should be pointed out that the observed analogies require a great number of similarities to exist between the granules containing the three enzymes, for the particular shape of an activation curve depends on a whole set of factors, which are not the same for each activating agent used. For instance, the osmotic activation curve reflects the statistical distribution of a group of properties which determine what is usually termed 'membrane resistance'. To these properties must be added the permeability of the membrane to NaCl, to account for the activation which obtains in salt solutions. On the other hand, an instrument such as the Waring Blendor may be expected to hit in a much more random manner, although even here some properties of the granules may be involved. Again, the susceptibility to freezing and thawing, pH or temperature must be dependent to a certain extent on inherent characteristics of the granules.

Barring the possibility of a chemical link between the three enzymes—a hypothesis which has been disproved experimentally, at least for acid phosphatase and β-glucuronidase—the most obvious conclusion is that they are actually associated within the same granules. This view, which has been put forward in a previous report (Duve *et al.* 1953), was rendered all the more likely by the finding, in preliminary experiments, that the distribution pattern of the two other hydrolases is similar to that

found for the acid phosphatase-containing granules. As will be shown in a subsequent paper (Appelmans, Wattiaux & Duve, 1955), these granules form a special group, entirely distinct from the oxidizing mitochondria and from the microsomes, and are probably represented by a relatively small number of individuals. Their cytological nature is unknown and any new information which could be obtained regarding their structure and enzymic equipment appeared therefore as particularly valuable. These facts have been commented upon also in a recent review (Duve & Berthet, 1954).

Experiments now in progress have thrown some doubt on the above interpretation, showing that the distribution of β-glucuronidase is not identical with that of acid phosphatase. The difference is slight, involving a shift of about 15% of β-glucuronidase from a lighter mitochondrial fraction to the microsomes, but it is reproducible and clearly beyond recognized experimental errors. Studies on tumours and precancerous tissues have also furnished indications that the two enzymes may behave in an independent manner. These investigations, which will be reported in later publications, must be pursued further before allowing definite conclusions. But they are mentioned in view of the important incidence they may have on the final interpretation of the results presented here.

SUMMARY

1. The β-glucuronidase and cathepsin of rat liver share a number of the properties previously described for acid phosphatase. Like this enzyme, they are enclosed within cytoplasmic granules and exert their full activity in an *in vitro* assay only if the granules have been subjected to a treatment which simultaneously releases the enzymes in soluble form.

2. The release of the three enzymes proceeds almost identically in washed particulate preparations activated either by the Waring Blendor, freezing and thawing or exposure to hypotonic media. Incubation at 0° in 0·15M-NaCl, or at 37° and pH 5 in 0·25M sucrose also causes a parallel liberation of β-glucuronidase and acid phosphatase. Cathepsin was not measured in these experiments.

3. Once released, acid phosphatase and β-glucuronidase are easily separable by means of ammonium sulphate fractionation. The partial purification of acid phosphatase is described.

4. In discussing the results obtained, it is concluded that the three enzymes behave essentially as though they were associated within the same granules. However, recent unpublished data suggest that this conclusion may have to be revised.

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Tissue Fractionation Studies

5. THE ASSOCIATION OF ACID PHOSPHATASE WITH A SPECIAL CLASS OF CYTOPLASMIC GRANULES IN RAT LIVER

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In a previous study of the intracellular distribution of acid phosphatase in rat liver, it was found that the bound form of the enzyme, although associated mainly with the mitochondria, is also recovered to some extent with the microsomes (Berthet & Duve, 1951). Similar observations were made by Palade (1951). Other mitochondrial enzymes, such as cytochrome oxidase, do not show this peculiarity, and it seemed of interest to ascertain the cause of the discrepancy. The investigations which were undertaken in order to clarify this point are reported in the present paper. They have led to the discovery that acid phosphatase is attached to a special type of cytoplasmic granules, differing both from the cytochrome oxidase-bearing mitochondria and from the glucose 6-phosphatase-containing micro-